

AD _____

Award Number: DAMD17-00-1-0345

TITLE: Genetic Determinants of Inflammatory Breast Cancer

PRINCIPAL INVESTIGATOR: Sofia D. Merajver, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040903 058

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Annual (15 Jun 2002 - 14 Jun 2003)	
4. TITLE AND SUBTITLE Genetic Determinants of Inflammatory Breast Cancer			5. FUNDING NUMBERS DAMD17-00-1-0345	
6. AUTHOR(S) Sofia D. Merajver, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Ann Arbor, MI 48109-1274 E-Mail: smerajve@umich.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Primary inflammatory breast cancer (IBC) accounts for approximately 6% of new breast cancers annually. We hypothesized that a limited number of concordant genetic alterations give rise to the unique aggressive inflammatory phenotype of IBC. While working on the genetic determinants underlying the IBC phenotype, we found concordant and consistent alterations of two genes, RhoC GTPase and a novel IGF-binding protein (IGF-BP), in patients with IBC. RhoC was overexpressed in 90% of IBC samples examined compared to 30% of stage matched non-IBC tumors. LIBC was lost in 80% of the IBC samples and only 20% of non-IBC samples examined. Since RhoC and LIBC appear to act in concert in IBC, coupled to the preliminary evidence from other laboratories of genes from these families playing a role in pancreatic cancer (another highly aggressive adenocarcinoma), they are excellent candidate genes to begin to probe the genetic basis of the aggressive phenotype in IBC. We hypothesize that the phenotype of IBC is due to alterations in expression of RhoC and IGF-BP early in tumorigenesis. We will elucidate the signaling pathway downstream from RhoC GTPase and attempt to determine what effect RhoC and LIBC have on cellular motility and invasion.				
14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) Motility, invasion, aggressive phenotype				15. NUMBER OF PAGES 11
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	6
References.....	7
Appendices.....	9

Introduction:

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer and accounts for approximately 6% of new breast cancer cases annually in the United States (1, 2). IBC has distinct clinical and pathological features. Patients present with erythema, skin nodules, dimpling of the skin (termed "peau d'orange"), all features that develop rapidly, typically progressing within 6 months (1-4). One salient feature of IBC that is observed in tissue sections is that cancer cells form emboli that spread through the dermal lymphatics. The dermatotropism of IBC is believed to be responsible for the clinical signs and symptoms and probably enables effective dissemination to distant sites (2). These observations lead us to conclude that IBC is highly invasive and it is capable of metastases from its inception. Indeed, at the time of diagnosis, the majority of patients have locoregional and/or distant metastatic disease (3, 4). In spite of new advances in breast cancer therapy including multimodality approaches, the 5-year disease-free survival rate is less than 45% (3, 4).

Until recently, no biologic markers defined the IBC phenotype. We hypothesized that a limited number of genetic alterations, occurring in rapid succession or concordantly, are responsible for the rapidly progressive and distinct clinical and pathologic features of IBC. Using a modified version of the differential display technique and *in situ* hybridization of human tumors, we identified two genes that are consistently and concordantly altered in human IBC when compared to stage-matched, non-IBC tumors: loss of WISP3 and over-expression of RhoC-GTPase (5).

WISP3 is member of the CCN family of proteins, which have important biological functions in normal physiology as well as in carcinogenesis (6-8). We found that WISP3 has growth and angiogenesis inhibitory functions in IBC *in vitro* and *in vivo* (9). RhoC-GTPase is a member of the Ras-superfamily of small guanosine triphosphatases (GTPases). Activation of Rho proteins leads to assembly of the actin-myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility (10-12). Our laboratory has characterized RhoC as a transforming oncogene for human mammary epithelial cells, whose overexpression results in a highly motile and invasive phenotype that recapitulates the IBC phenotype. Predicated on the high rate of concordance of RhoC and WISP3 changes in IBC, we hypothesize that these two genes cooperate to determine this highly metastatic, unique breast cancer phenotype.

This is an annual progress report for a project that aims at understanding the genetic determinants of inflammatory breast cancer (IBC). In particular, we aim to discern the relative role of the RhoC GTPase gene and WISP3 (formally named LIBC) in the specific phenotypic characteristics of inflammatory breast cancer. We have made progress in the last year, which is summarized below.

Body:**Task 1: To identify the signaling pathways involved in growth control affected by RhoC overexpression or mutation in inflammatory breast cancer.**

We have completed the majority of Task 1 and are currently trying to understand the relationship between RhoC and cyclinD1/pp125FAK. We encountered some difficulty with the quality of antibodies, specifically cyclinD1 and RhoC, but now these issues are resolved and progress is underway. There is no reportable outcome on this task for this report but we are confident that in our next progress report we will have completed this task.

Task 2: To explore the role of RhoC and IGF-binding proteins in IGF-induced motility and invasion.

To begin to address the experimental questions in Task 2, we had to develop cell lines that have high RhoC expression and low LIBC (WISP3) expression. We decided to use immortalized mammary epithelial cells (HME) as our model cell line. Using an antisense approach, inhibition of WISP3 expression in HME cells resulted in a 3-fold increase in RhoC GTPase transcript levels (Figure 1). The HME/high RhoC-low WISP3 cells exhibited increased cellular proliferation and anchorage independent growth in soft agar (Figure 2). These high RhoC-low WISP3 clones produced significantly more colonies in soft agar when compared with the control cells, an average of 58% of the level of colonies formed by the SUM149 IBC cells. Moreover, these HME/high RhoC-low WISP3 cells also exhibited decreased production of VEGF in the conditioned medium (Figure 2). Consistent with Task 2, our ongoing efforts are to address the differential motility and invasion of these clones.

Specific Response to Reviewer:

We have not or are not planning to screen for farnesyl transferase inhibitors of RhoC GTPase. However, in a published study (van Golen et al., Mol. Cancer Ther., 2002), we assessed the effect of a farnesyl transferase inhibitor, FTI L-744,832, on RhoC-overexpressing IBC and RhoC-transfected human mammary epithelial (HME-RhoC) cells. Treatment of the SUM149 IBC cell line and HME-RhoC transfectants with the FTI L-744,832 led to reversion of the RhoC-induced phenotype, manifested by a significant decrease in anchorage-independent growth, motility, and invasion.

Key Research Accomplishments and Reportable Outcomes:

1. WISP3 was found to directly modulate RhoC GTPase expression in immortalized mammary epithelial cells.
2. Immortalized mammary epithelial cells with low WISP3 and high RhoC GTPase displayed neoplastic characteristics, specifically, an increase in cell proliferation,

an increase in anchorage independent growth, and an increase in VEGF production.

Conclusions:

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer, with a 5-year disease free survival of less than 45%. Our work focused on determining the genetic alterations that result in this aggressive breast cancer phenotype. Previously, we have found that RhoC and WISP3 are consistently and concordantly altered in IBC tissues. RhoC functions as an oncogene, and WISP3 as a tumor suppressor gene. Here, we provide evidence that supports the hypothesis that these two genes act in concert to give rise to the highly aggressive IBC phenotype.

References:

1. Jaiyesimi, I. A., Buzdar, A. U., and Hortobagyi, G. Inflammatory breast cancer: a review. *J Clin Oncol*, 10: 1014-1024., 1992.
2. Lee, B. J. a. T., N.D. Inflammatory carcinoma of the breast: a report of twenty-eight cases from the breast clinic of Memorial Hospital. *Surg Gynecol Obstet*, 39: 580-595, 1924.
3. Merajver, S. D., Weber, B. L., Cody, R., Zhang, D., Strawderman, M., Calzone, K. A., LeClaire, V., Levin, A., Irani, J., Halvie, M., August, D., Wicha, M., Lichter, A., and Pierce, L. J. Breast conservation and prolonged chemotherapy for locally advanced breast cancer: the University of Michigan experience. *J Clin Oncol*, 15: 2873-2881., 1997.
4. Swain, S. M., Sorace, R. A., Bagley, C. S., Danforth, D. N., Jr., Bader, J., Wesley, M. N., Steinberg, S. M., and Lippman, M. E. Neoadjuvant chemotherapy in the combined modality approach of locally advanced nonmetastatic breast cancer. *Cancer Res*, 47: 3889-3894., 1987.
5. van Golen, K. L., Davies, S., Wu, Z. F., Wang, Y., Bucana, C. D., Root, H., Chandrasekharappa, S., Strawderman, M., Ethier, S. P., and Merajver, S. D. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. *Clin Cancer Res*, 5: 2511-2519., 1999.
6. Perbal, B. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. *Mol Pathol*, 54: 57-79., 2001.
7. Pennica, D., Swanson, T. A., Welsh, J. W., Roy, M. A., Lawrence, D. A., Lee, J., Brush, J., Taneyhill, L. A., Deuel, B., Lew, M., Watanabe, C., Cohen, R. L., Melhem, M. F., Finley, G. G., Quirke, P., Goddard, A. D., Hillan, K. J., Gurney, A. L., Botstein, D., and Levine, A. J. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci U S A*, 95: 14717-14722., 1998.
8. Hurvitz, J. R., Suwairi, W. M., Van Hul, W., El-Shanti, H., Superti-Furga, A., Roudier, J., Holderbaum, D., Pauli, R. M., Herd, J. K., Van Hul, E. V., Rezai-Delui, H., Legius, E., Le Merrer, M., Al-Alami, J., Bahabri, S. A., and Warman, M. L. Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat Genet*, 23: 94-98., 1999.
9. Kleer, C. G., Zhang, Y., Pan, Q., van Golen, K. L., Wu, Z. F., Livant, D., and Merajver, S. D. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. *Oncogene*, 21: 3172-3180, 2002.
10. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*, 273: 245-248, 1996.
11. Leung, T., Chen, X. Q., Manser, E., and Lim, L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol Cell Biol*, 16: 5313-5327, 1996.

12. Nobes, C. D. and Hall, A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, 81: 53-62, 1995.

Appendices

Figures 1-2

Figure 1

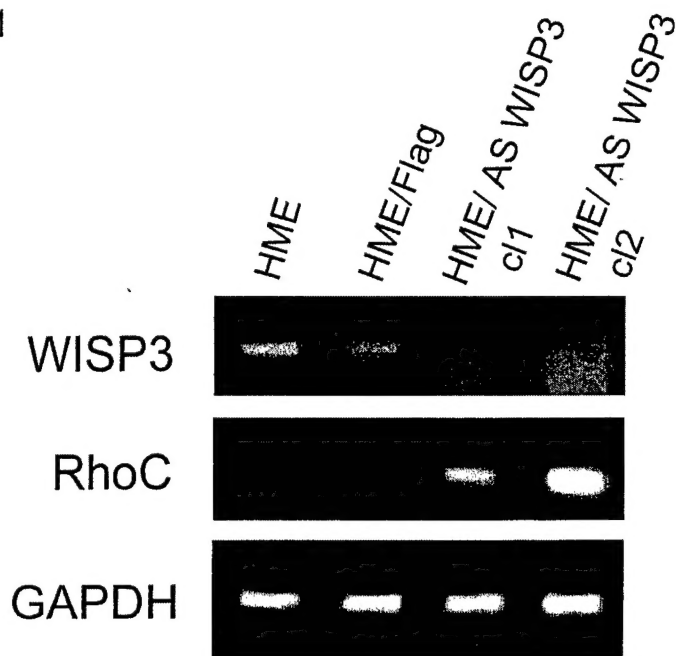
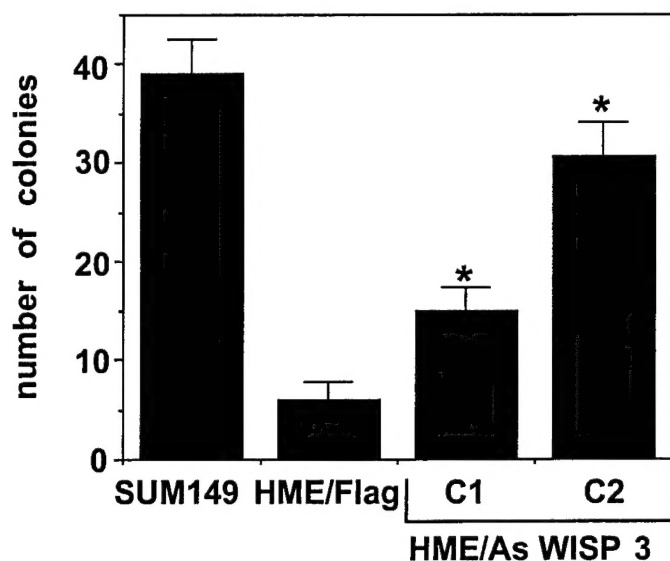


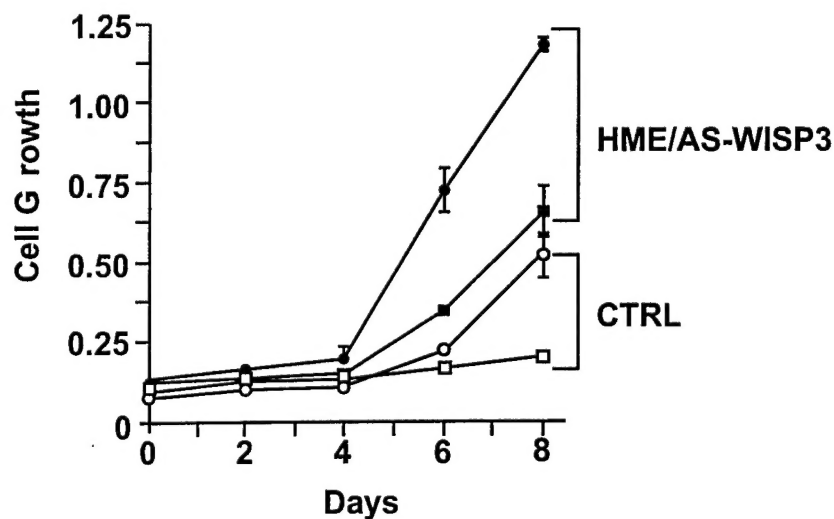
Figure 1. Inhibition of WISP3 in HME cells results in an increase in RhoC transcript levels. RT-PCR was conducted on vector and HME cells that have inhibition of WISP3 expression using full-length WISP3 antisense mRNA. HME/ AS WISP3 cells demonstrated increased levels of RhoC transcript compared to controls.

Figure 2

A



B



C

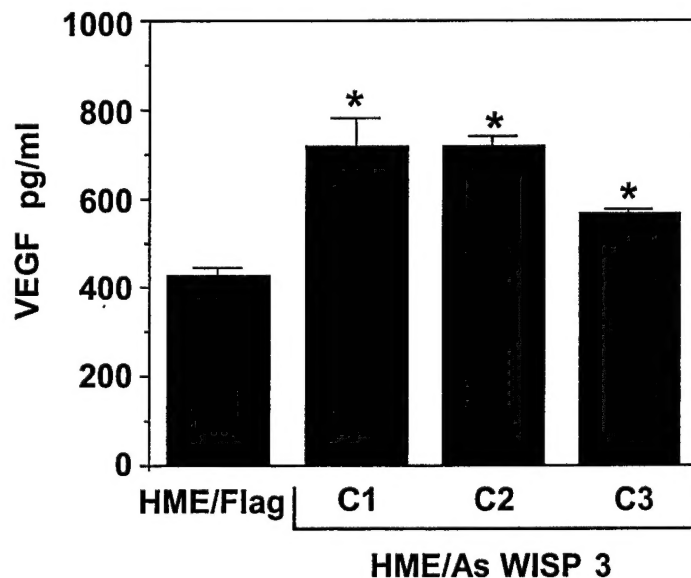


Figure 2. Inhibition of WISP3 induces anchorage-independent growth, proliferation and secretion of VEGF in HME cells. (A) Inhibition of WISP3 expression in HME cells greatly increased the number of colonies formed in soft agar when compared to empty vector control (HME/ Flag); t test, $p < 0.05$. (B) Effect of inhibition of WISP3 expression on the proliferation of HME cells was studied with the MTT assay. The stable HME/ AS WISP3 cells have a significant increase in the proliferation rate when compared to the empty vector control. Results are expressed as mean \pm SEM of three independent experiments; t test, $p < 0.05$. (C) Increase in VEGF measured by ELISA, as a result of inhibition of WISP3 expression in HME cells. Results are expressed as mean \pm SEM; t test, $p < 0.05$.